AD_____

Award Number: DAMD17-03-1-0524

TITLE: Overcoming Bone Marrow Stroma-Mediated Chemoresistance in Metastatic Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Robert Wieder, M.D., Ph.D.

CONTRACTING ORGANIZATION: New Jersey Medical School
Newark, NJ 07101-1709

REPORT DATE: August 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-08-2005	Annual	7 Jul 2004 – 6 Jul 2005

4. TITLE AND SUBTITLE
Overcoming Bone Marrow Stroma-Mediated Chemoresistance in Metastatic Breast Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0524

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert Wieder, M.D., Ph.D.

E-mail: wiederro@umdnj.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New Jersey Medical School
Newark, NJ 07101-1709

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Well-differentiated breast cancer cells metastasize to the bone marrow where they can remain dormant for years and are resistant to chemotherapy. We have developed an in vitro model where well-differentiated breast cancer cell lines treated with FGF-2, a growth factor abundant in the marrow microenvironment, stop proliferating, partially re-differentiate by re-expressing novel integrins such as alpha 5 beta 1 though which they ligate bone marrow fibronectin and receive survival signaling. This signaling includes sustained activation of the PI3 kinase/Akt pathway. Here, we demonstrate that all trans-retinoic acid (ATRA) inhibits Akt phosphorylation and inhibits survival of dormant clones in a dose-dependent manner at concentrations achievable in vivo. Cells treated with ATRA lose the characteristic morphology of these dormant cellsand appear stellate and shrunken. We investigated the effects o the small GTPase Rho, with reported roles in cell spreading. Blocking Rho and its downstream kinase ROCK inhibited survival of dormant clones in a dose-dependent manner. Combined blockade of PI3 kinase and Rho or ROCK achieved a synergistic inhibition of dormant clone survival, resulting in their nearly total abrogation. These data suggest that combined blockade of these signaling pathways may merit study of their effects on eliminating dormant breast cancer cells in the marrow.

15. SUBJECT TERMS
Dormancy, integrins fibronectin, metastasis, Akt, PI3 kinase, all trans-retinoic acid (ATRA)

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	12	

Table of Contents

INTRODUCTION

Objectives/Hypothesis:

A. At the time of diagnosis, even early stages of breast cancer have microscopic metastases in the bone marrow. These microscopic metastases are resistant to adjuvant chemotherapy. The mechanisms of this resistance are not understood. In a model developed in our laboratory, fibroblast growth factor 2 (FGF-2), present in large deposits in the bone marrow stroma, inhibits proliferation of well-differentiated breast cancer cells. In turn, FGF-2 also induces a redifferentiation of these cells that includes an increased expression of integrins $\alpha 5$ and $\beta 1$. Through integrins $\alpha 5$ and $\beta 1$, the cells bind fibronectin and activate survival signaling through the PI3 kinase/Akt pathway (1).

Reports have been published that derivatives of retinoic acid can inhibit the activation of Akt in another system. We and others have demonstrated that all trans-retinoic acid inhibits the proliferation and induces apoptosis in breast cancer cells (2). We hypothesized that all *trans*-retinoic acid (ATRA) would inhibit Akt activation in dormant clones and abrogate their survival. We carried out experiments to test this hypothesis.

B. In our original study of the dormancy model of well-differentiated breast cancer cells, blocking the PI3K/Akt pathway did not completely eliminate the dormant clones, suggesting the involvement of additional signal pathways (1). Indeed, blocking ERK and p38 signaling individually also inhibited but did not eliminate survival of these dormant clones (3). While these cells were resistant to doses of the chemotherapy agent paclitaxel up to one hundred-fold higher than those sufficient to eliminate growing clones, they could be abrogated by flavopiridol, a drug that has pleiotropic effects.

Insight to an important process in survival of the dormant clones was provided by inhibition studies with the PI3K inhibitor LY294002. The surviving clones lost their spread out appearance and assumed a stellate, distressed morphology, suggesting the involvement of small GTPases and focal adhesion activation in the surviving cells. One of the mechanisms involved in survival of these dormant clones may involve maintenance of the spread phenotype. The small GTPase RhoA is necessary for cell adhesion and spreading (4). Inhibition of RhoA with C3 transferase or blocking membrane localization by inhibiting geranylgeranylation, or inhibition of Rho kinase (ROCK), a target kinase of RhoGTP results in cell death in endothelial cells (5). ATRA can inhibit both the expression and the GTP-loading of RhoA (6). While inhibition of RhoA or ROCK does not affect the PI3K pathway (5), reports put PI3K upstream of RhoA (7, 8). PI3K is permissive for Rho GTP loading by the ineffective interaction of the p85 subunit with the RhoGAP binding site of Rho GTPases (9). The p85 activating subunit can also activate Rho GTPases by interacting directly with guanine nucleotide exchange factors (GEFs) (10). Based on these facts, we investigated the role of RhoA and its relationship to PI3K signaling in the survival of these dormant cells.

We hypothesized that additional signaling involving Rho GTPases may be involved in survival of dormant clones and may coincide with PI3K-mediated survival signaling.

Study design:

A. Two well-differentiated breast cancer cell lines, MCF-7 and T-47D were used in the studies. Cells were incubated in DMEM/10% fetal calf serum (FCS) with FGF-2 10 ng/ml in fibronectin-coated tissue culture plates (Biocoat, Becton Dickinson, MA) at clonogenic densities (1000

cells/well in 24 well plates for T-47D, 2000 cells/well for MCF-7 cells). At this plating density, cells do not come in contact with each other and primary interaction is with the substratum. After three days of culture, the medium was replaced with DMEM/5% charcoal stripped FCS and all trans-retinoic acid (ATRA) was added at concentrations of 0 (DMSO 1:10000 control), 10^{-9}, 10^{-8} and 10^{-7} M for an additional three or six days. Cells were fixed and stained with crystal violet solution containing sodium borate and ethanol. Colonies of 2-10 cells (dormant clones) were counted. In parallel experiments, after three day in culture or an additional three days in ATRA, cells were collected and lysates were prepared and analyzed by Western blot.

B. In experiments set up as described in A, after three days of culture, the medium and FGF-2 were replenished and appropriate inhibitors wee added. These were C3 transferase, a bacterial protein that inhibits RhoGTP (Cytoskeleton, Inc. Denver, CO), ROCK inhibitor (Santa Cruz Biotechnology, Inc), the PI3 kinase inhibitor Ly294002 and Akt inhibitor (Calbiochem, LaJolla, CA). Cells were fixed and stained with crystal violet solution containing sodium borate and ethanol. Colonies of 2-10 cells (dormant clones) were counted. In parallel experiments, lysates were prepared on day four, one day after the addition of C3 transferase. RhoGTP was precipitated with a Rhotekin-agarose slurry (UBS, Waltham, MA) and analyzed by Western blot with an anti-Rho antibody. Cellular lysates were also analyzed by Western blot for total Rho as a loading control. Cells were treated with the Akt inhibitor and analyzed by Western blot with an antibody to phospho-Akt.

BODY

A. FGF-2 induced the upregulated expression of integrins $\alpha 5$ and $\beta 1$ protein levels as demonstrated by Western blots after three and six days in sparse culture on fibronectin. This was associated with an increased level of phosphorylation of the protein kinase Akt. The increased phosphorylation of Akt was observed for three days in culture and was sustained for the six days assayed (Figure 1A, shown are T-47D cells). To determine if the phosphorylation of Akt resulted in its activation, we determined the phosphorylation status of GSK3, a target kinase downstream of Akt. Incubation of cells with FGF-2 on fibronectin for six days resulted in phosphorylation of Ser21/9 of GSK-3alpha/beta isoforms (Figure 1B). This confirmed that FGF-2 was inducing the activation of Akt.

Figure 1.

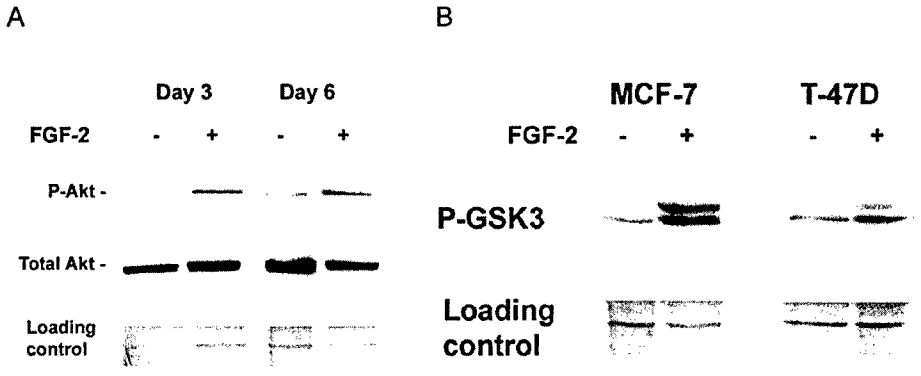


Figure 1. MCF-7 and T-47D cells were incubated at clonogenic density on fibronectin with and without FGF-2 for three or 6 days. Lysates were assayed by Western with phospho-Akt antibody (A, shown are MCF-7 cells) or with antibody to phosphorylated Ser21/9 of GSK-3alpha/beta isoforms (shown is the 6 day blot). Non-specific bands on Coomasie blue-stained gels were used to determine loading.

To determine if ATRA was able to affect the phosphorylation of Akt in this system, we analyzed cells incubated with FGF-2 on fibronectin with and without variable concentrations of ATRA. Cells were incubated with FGF-2 for three days. The medium was replaced with DMEM/5% charcoal-stripped FCS with FGF-2 and ATRA at variable concentrations and lysates were analyzed by Western blot. Figure 2A demonstrates that FGF-2 sustained the phosphorylation of Akt for six days while ATRA inhibited this phosphorylation. We wanted to determine if the observed inhibition of Akt phosphorylation by ATRA was associated with a modulation of the upregulated expression of integrins $\alpha 5$ and $\beta 1$. We analyzed the lysates obtained in the above experiments for expression of these integrins. Figure 2B demonstrates that ATRA did not affect the increased expression of integrins $\alpha 5$ and $\beta 1$ induced by FGF-2 in MCF-7 cells. We concluded that ATRA-mediated inhibition of Akt phosphorylation was not due to the suppression of integrin $\alpha 5$ and $\beta 1$ expression.

Figure 2.

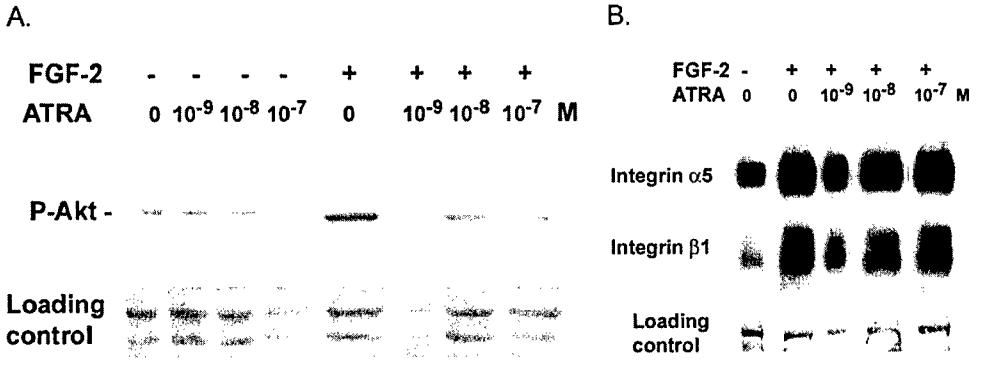


Figure 2. A. ATRA inhibited Akt Ser^{473} phosphorylation induced by FGF-2 in dormant clones. T-47D cells were incubated at clonogenic density on fibronectin with and without FGF-2 for three days and ATRA was added for an additional 3 days at the concentrations shown. Lysates were assayed by Western with phospho-Akt antibody. Non-specific protein bands on stained membranes were used as a loading control. B. ATRA does not affect FGF-2-mediated integrin upregulation. The lysates prepared in A were analyzed by Western blot with antibodies to integrins $\alpha 5$ and $\beta 1$, as above. Non–specific bands were used as loading controls.

In order to determine if the inhibition of Akt phosphorylation was associated with a phenotypic effect on dormant clones, MCF-7 and T-47D cells were incubated for three days with FGF-2, followed by ATRA treatment at the variable concentrations noted above for an additional three to six days. ATRA inhibited the survival of dormant clones in a dose dependent manner after three and six (Figure 3A) days of incubation. The appearance of the surviving clones in ATRA was significantly altered from that of the control dormant clones. While the dormant cells were large, well spread with large cytoplasm to nucleus ratios, the ATRA-treated cells were sickly appearing dendritic shapes that gave the appearance of extreme cellular distress. These data suggested that ATRA was inhibiting dormant clone survival in part by inhibiting the Akt pathway but also in part by disrupting mechanism responsible for cell spreading. The following studies investigated the role of Rho GTPase in survival of dormant breast cancer cells.

These data demonstrated that the upregulated expression of integrins $\alpha 5$ and $\beta 1$ and the activation of Akt were sustained during the six days of assay of dormant clones in the two well differentiated breast cancer cell lines tested. ATRA inhibited the activation of Akt in dormant clones at concentrations beginning at 10^{-9} M and significantly inhibited the survival of dormant clones in a dose-dependent manner. These data suggest that ATRA, which is currently being tested in clinical trials in breast cancer, may have a potential value in the treatment of resistant micrometastases

Figure 3.

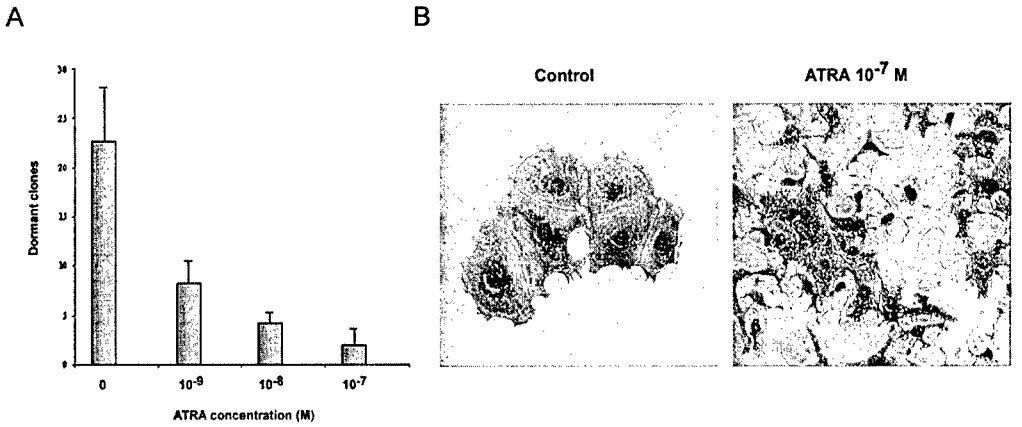


Figure 3. A. ATRA inhibits survival of dormant T-47D clones. Cells were incubated at clonogenic density on fibronectin with FGF-2. After 3 days the media and FGF-2 were replaced and media containing 5% charcoal stripped fetal calf serum with variable concentrations of ATRA added. The media and supplements were refreshed again on day 6 and dormant colonies of ≤ 10 cells were stained and counted on day 9. B. The appearance of control and ATRA-treated dormant clones on day 9.

B. We tested the hypothesis signaling involving Rho GTPase may be involved in survival of dormant clones. The RhoGTP activation assay demonstrated that in dormant cells incubated with FGF-2 on fibronectin, C3 tansferase 5 μg/ml reduced the GTP loading of Rho (Figure 4A). Total Rho levels were unaffected. In experiments aimed at determining the effects of inhibiting Rho and its downstream target, Rho kinase, on the survival of dormant clones, results demonstrated that C3 transferase and ROCK inhibitor both reduced the number of surviving dormant clones in a dose-dependent manner in T-47D cells (Figure 4B).

Figure 4.

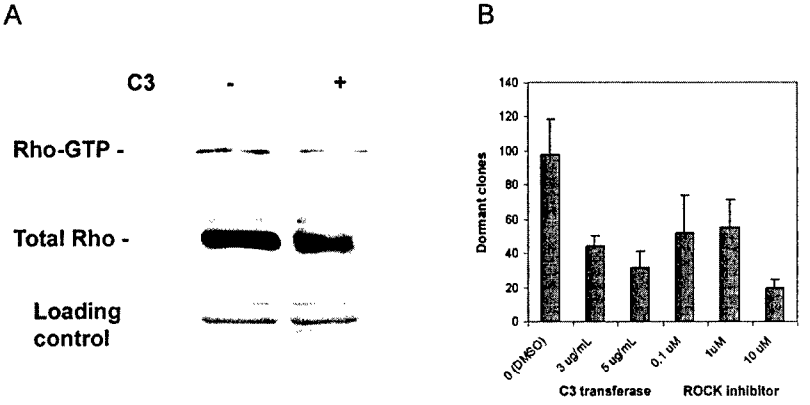


Figure 4. A. C3 transferase inhibits RhoA GTP loading. T-47D cells were incubated at clonogenic density on fibronectin with FGF-2, FGF-2 was replaced after 3 days and C3 transferase (Cytoskeleton, Inc., Denver, CO) 5 μg/ml was added for 24 hours. RhoGTP loading was assayed by Rhotekin-agarose pull down assay (UBS) and analyzed by Western with anti-Rho antibody. Total Rho was assayed in total cell lysates. B. Dose-dependent inhibition of dormant clones by C3 transferase, or the ROCK inhibitor Y27632.

In this system, Akt inhibitor 25 μM reduced the FGF-2-induced Ser^{473} phosphorylation of Akt in cells treated with FGF-2 10 ng/ml (Figure 5A and B). Inhibition of Akt and of PI3 kinase, the upstream activator of Akt, resulted in a dose-dependent reduction in the survival of dormant clones (Figure 5C).

Figure 5.

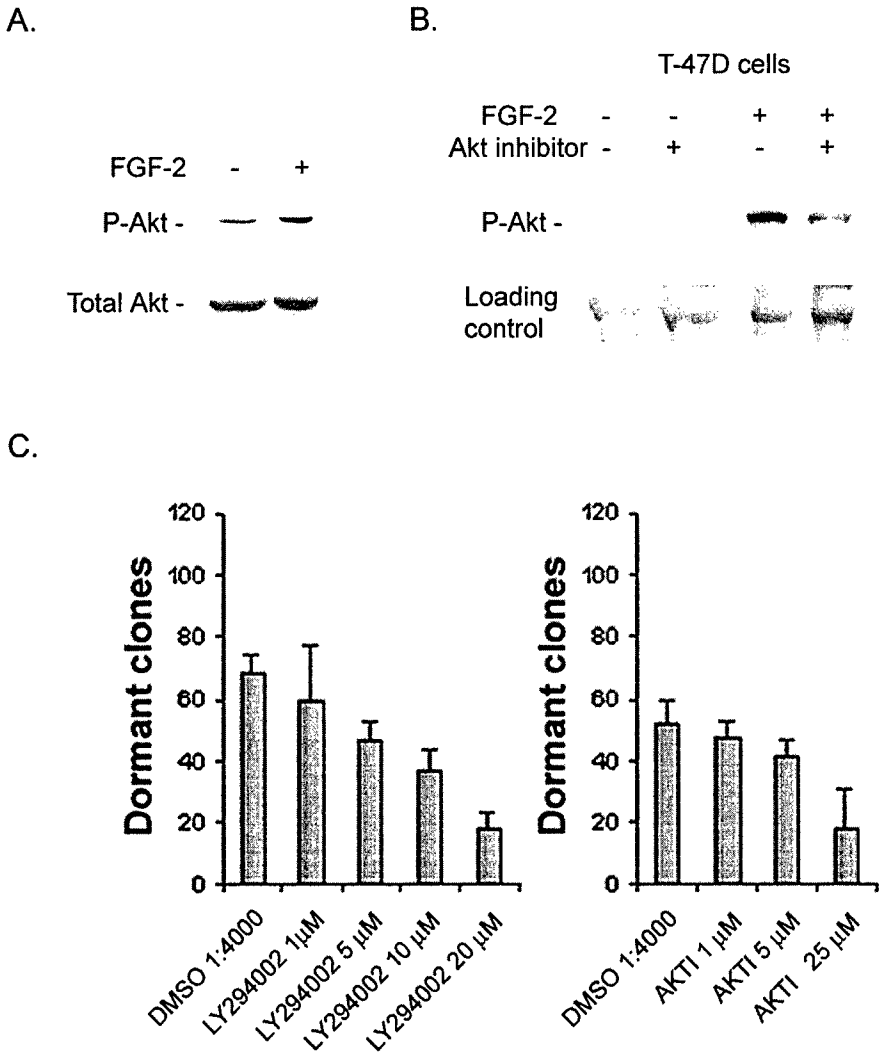
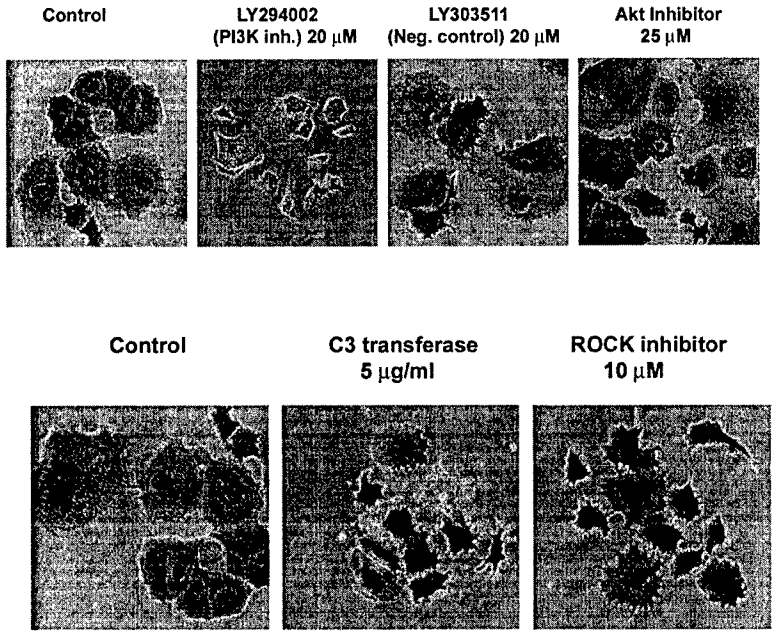


Figure 5. A. Western blot of lysates from T-47D cells incubated on fibronectin with and without FGF-2 for up to 5 days (3 days shown) were stained with antibody to phospho-Akt or total Akt. FGF-2 induced sustained Akt phosphorylation in T-47D cells (and MCF-7 cells, not shown). B The induction of Ser^{473} phosphorylation of Akt by a 4 day incubation with FGF-2 10 ng/ml was inhibited by a 24 hour incubation with Akt inhibitor 25 μM added on day 3. C. Dormant clones incubated with FGF-2 for 6 days were inhibited in a dose-dependent manner by addition of Akt inhibitor and the PI3 kinase inhibitor LY294002 on day 3.

The morphology of the surviving clones following treatment with the various inhibitors was impressive. Control dormant clones consisted of cells that were large, flat and spread out with large cytoplasm to nucleus ratios. Treatment with Akt inhibitor did not affect cell morphology. However, cells in clones that survived either C3 transferase or ROCK inhibitor or LY294002 were small, dendritic and appeared distressed and dying (Figure 6A). The fact that both PI3K and Rho

inhibition affected surviving clone morphology suggested that the to molecules may be interdependent in providing survival to the dormant cells. We tested this hypothesis by combining inhibitors of the two pathways. When C3 transferase and the PI3 kinase inhibitor LY294002 were combined, there was an almost complete obliteration of dormant clone survival (Figure 6B). Similar data were obtained with the combination of the ROCK inhibitor and LY294002 (not shown).

Figure 6.
A.



B.

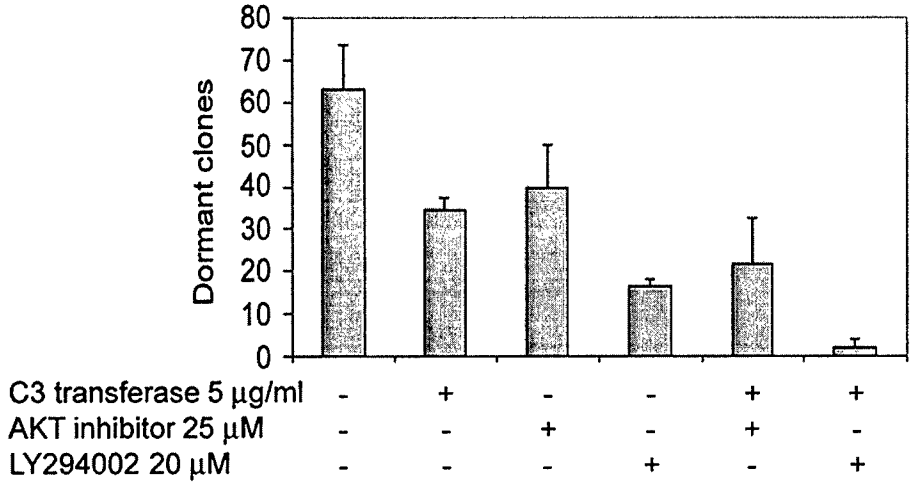


Figure 6. A. The appearance of surviving dormant T-47D clones after treatment with PI3K inhibitor LY294002, negative control LY303511 and Akt inhibitor (Calbiochem), C3 transferase and ROCK inhibitor Y27632 (Santa Cruz). B. Combined effects of RhoA inhibitor and Akt inhibitor or PI3 kinase inhibitor on survival of dormant T-47D clones. T-47D cells were incubated at clonogenic density of 1000 cells per well on 24 well fibronectin-coated plates with FGF-2 10 ng/ml for 6 days. C3 transferase 5 μg/ml, Akt inhibitor 25 μM and LY294002 20 μM were added individually or in combination on day 3 and dormant clones were counted on day 6.

These data demonstrate that the inhibition of Rho-GTP and Rho kinase inhibits the survival of dormant breast cancer cell clones in a tissue culture model in a dose dependent manner. In addition, inhibition of PI3 kinase and Akt also inhibits dormant clone survival in a dose dependent manner. Combining the inhibition of the two pathways almost completely eradicates dormant clones, suggesting both pathways contribute to survival. Therapy targeted at these pathways may overcome chemoresistance of microscopic metastases.

KEY RESEARCH ACCOMPLISHMENTS

1. Upregulated expression of integrins $\alpha 5$ and $\beta 1$ and activation of Akt are sustained during the six days of assay of dormant clones in two well-differentiated breast cancer cell lines tested.
2. ATRA inhibits activation of Akt in dormant clones at 10^{-9} M concentrations.
3. ATRA significantly inhibits survival of dormant clones in a dose-dependent manner.
4. ATRA treatment significantly impairs the spread appearance of dormant cells.
5. Inhibition of Rho GTP-loading and of Rho kinase inhibits the survival of dormant breast cancer cell clones in a dose dependent manner.
6. Inhibition of PI3 kinase and Akt also inhibits dormant clone survival in a dose dependent manner. However, the spread morphology depends on PI3K but not on Akt signaling.
7. Combined inhibition of PI3K and Rho or ROCK almost completely eradicates dormant clones but combined inhibition of Akt and Rho or ROCK is not additive. This suggests that PI3K and Rho are interdependent while Akt and Rho have branched effects on survival. Therapy targeted at these pathways may overcome chemoresistance of microscopic metastases.

REPORTABLE OUTCOMES

Korah R, Choi L, Barrios J and Wieder R. (2004) Constitutive expression of FGF-2 abrogates focal adhesion signaling in MDA-MB-231 breast cancer cells. Breast Cancer Research and Treatment 88: 17-28 (Erratum – color photos (2005) 89: 319 – 322).

Najmi S, Korah R, Chandra R, Abdellatif M, Wieder R. (2005) Flavopiridol blocks integrin-mediated survival in dormant breast cancer cells. Clinical Cancer Research 11:2038-2046.

A. Shah, R. Korah and R. Wieder. FGF-2 suppresses EGF-mediated T-47D breast cancer cell motility. Proceedings of the American Association for Cancer Research 2003, v. 44, 2nd ed., p. 869, #4384.

Ankoor Shah, Reju Korah and Robert Wieder. FGF-2 inhibits motility response to EGF in T-47D breast cancer cells. (2003) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, p. 68, # P59.

Mike Lindy, Reju Korah, Monika Boots, and Robert Wieder. Role of RhoA in survival of dormant breast cancer cells by basic fibroblast growth factor. Proceedings of the American Association for Cancer Research 2004, v. 45, #2435 p. 563 (oral presentation).

Michael E. Lindy, Reju Korah, Monika Boots, and Robert Wieder. Survival of dormant breast cancer cells depends on PI3 kinase and Rho signaling. (2004) The National Student Research Forum, The University of Texas Medical Branch, Galveston, TX. (selected for oral presentation).

Joseph, V, Wieder, R. All trans-retinoic acid modulates survival signaling in dormant breast cancer cells. Program Proceedings of American Society of Clinical Oncology, 2004, v. 23. # 422.

Korah R, Lindy M, Boots M, B. Benn and Wieder R. Interactions with the bone marrow microenvironment contribute to survival of breast cancer cells in a dormancy paradigm. Keystone Symposia on Microenvironment in tumor induction, Banff Centre, Alberta, Canada, February, 2005, p. 74, #333.

Robert Wieder, Saltanat Najmi, Rachna Chandra, Maha Abdellatif, Reju Korah. Flavopiridol disrupts adhesion and survival signaling in taxane-resistant dormant breast cancer cells. Proceedings of the American Association for Cancer Research 2005, v. 46, #5927, p.1394.

S Najmi, R Korah, M Abdellatif, R Wieder. Use of flavopiridol to disrupt adhesion and survival in dormant breast cancer cells. (2005) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, #116.

R. Wieder, M. Lindy, M. Boots, R. Korah . Survival signaling in dormant breast cancer cells. The Department of Defense Breast Cancer Research Program Meeting, "Era of Hope", June 2005, Philadelphia, p. 416, #P59-22.

CONCLUSIONS

1. Survival of dormant clones is mediated at least in part through the PI3 kinase/Akt pathway and the small GTPase Rho. Combined blockade of PI3K and Rho may be effective in eliminating dormant breast cancer clones.

2. All *trans*-retinoic acid (ATRA) downregulates the activated state of Akt in dormant cells and inhibits their survival in a dose-dependent manner at doses achievable under physiologic conditions. Morphologic changes in surviving cells suggest additional effects of ATRA on molecules that affect cell spreading, also involved in dormant cell survival.

REFERENCES

1. Korah R, Boots M, and Wieder R. (2004) Integrin $\alpha5\beta1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. Cancer Research 64: 4514-4522.
2. Wang Q, Yang W, Uytingco MS, Christakos S and Wieder R. (2000) 1,25$(OH)_2$ vitamin D_3 and all-*trans* retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. Cancer Research. 60:2040-2048.
3.Najmi S, Korah R, Chandra R, Abdellatif M, Wieder R. (2005) Flavopiridol blocks integrin-mediated survival in dormant breast cancer cells. Clinical Cancer Research 11:2038-2046.
4. Brancolini C, Marzinotto S, Edomi P, Agostoni E, Fiorentini C, Muller HW, and Schneider C. (1999) Rho-dependent regulation of cell spreading by the tetraspan membrane protein Gas3/PMP22. Molecular Biology of the Cell. 10:2441-2459.
5. Li X, Liu L, Tupper JC, Bannerman DD, Winn RK, Sebti SM, Hamilton AD, and Harlan JM. (2002) Inhibition of protein geranylgeranylation and RhoA/RhoA kinase pathway induces apoptosis in human endothelial cells. J. Biol. Chemistry. 277:15309-15316.

6. Langlois A, Lee S, Kim DS, Dirks PB, and Rutka JT. (2002) p16(ink4a) and retinoic acid modulate rhoA and GFAP expression during induction of a stellate phenotype in U343 MG-A astrocytoma cells. GLIA. 40:85-94.
7. Murakami H, Iwashita T, Asai N, Iwata Y, Narumiya S, and Takahashi M. (1999) Rho-dependent and -independent tyrosine phosphorylation of focal adhesion kinase, paxillin and p130Cas mediated by Ret kinase. Oncogene. 18:1975-1982.
8. Kim BC, Lee MN, Kim JY, Lee SS, Chang JD, Kim SS, Lee SY, and Kim JH. (1999) Roles of phosphatidylinositol 3-kinase and Rac in the nuclear signaling by tumor necrosis factor-alpha in rat-2 fibroblasts. J. Biol. Chemistry. 274:24372-24377.
9. Fidyk NJ, and Cerione RA. (2002) Understanding the catalytic mechanism of GTPase-activating proteins: demonstration of the importance of switch domain stabilization in the stimulation of GTP hydrolysis. Biochemistry. 41:15644-15653.
10. Innocenti M, Frittoli E, Ponzanelli I, Falck JR, Brachmann SM, Di Fiore PP, and Scita G. (2003) Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. J. Cell Biology. 160:17-23.